REMARKS/ARGUMENTS

Claims 70 to 155 remain in this application. Claims 1 to 69 have been canceled without prejudice to subsequent revival. Claims 70 to 155 have been added and include no new matter. New claims 70 to 155 are submitted for the purpose of advancing the case toward allowance and differences between the new claims and canceled claims should not be viewed as acquiescence to any of the Examiner's rejections.

The Examiner rejected the pending claims under 35USC112, first paragraph, stating that the claims fail to comply with the written description requirements. The Examiner states that the claims relate to a hybrid lysozyme gene comprising elements (a) to (f) and are therefore indefinite. Applicant traverses the rejection. However, the presently pending independent claims do not include elements (a) to (f) thereby obviating the Examiner's rejection.

The Examiner rejects the pending claims under 35USC112, second paragraph, stating that there is insufficient description in the prior art and instant specification of the changes that can be made to SEQ ID NO: 67 and still retain the recited functionality. The Examiner states that it would be remedial to state explicit hybridization conditions that would provide a nexus between structural and functional characteristics of the claimed nucleic acids. Applicant traverses this rejection. However, the presently pending claims specify "hybridization in the presence of about 1.0 M Na ion at a temperature of about 60° C" as is disclosed on page 24, lines 9 to 13. Therefore, applicant submits that the present rejection should be withdrawn.

Under 35USC112, second paragraph, the Examiner rejects certain claims stating that the term "intrinsically curved" does not appear to be explicitly defined in the specification or in the prior art. Applicant traverses this rejection.

Applicant submits that the term "intrinsically curved" DNA was well known in the art and understood by a practitioner of ordinary skill at the time of filing the present application. For example, it is well known that intrinsically curved DNA is typically AT rich DNA showing a curved shape and is a specific target site for protein binding. In addition, it is also well known the intrinsically curved DNA is often found in the promoter regions of DNA. Numerous references use the term "intrinsically curved" when referring to such DNA sequences. See, for example, EMBO (1997) Vol 16 No 7 pp 1795-1805, a copy of which is included with this RCE. Therefore, applicant respectfully submits that the term "intrinsically curved" DNA is understood in the art and that the present rejection is improper and should be withdrawn.

In conclusion, applicant has shown that the present claims satisfy the requirements of 35USC112, first and second paragraphs. Therefore, applicant submits that the presently

pending claims are allowable and respectfully requests the Examiner to pass the above-identified application to allowance at an early date.

Respectfully submitted,

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The oligomeric structure of nucleoid protein H-NS is necessary for recognition of intrinsically curved DNA and for DNA bending

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- Abstract
- Introduction
- Results
- Discussion
- Materials and methods
- Footnotes
- Acknowledgements
- References

Abstract

Escherichia coli hns, encoding the abundant nucleoid protein H-NS, was subjected to site-directed mutagenesis either to delete Pro115 or to replace it with alanine. Unlike the wild-type protein, hyperproduction of the mutant proteins did not inhibit macromolecular syntheses, was not toxic to cells and caused a less drastic compaction of the nucleoid. Gel shift and ligase-mediated circularization tests demonstrated that the mutant proteins retained almost normal affinity for non-curved DNA, but lost the wild-type capacity to recognize preferentially curved DNA and to actively bend non-curved DNA, a property of wild-type H-NS demonstrated here for the first time. DNase I footprinting and *in vitro* transcription experiments showed that the mutant proteins also failed to recognize the intrinsically bent site of the *hns* promoter required for H-NS transcription autorepression and to inhibit transcription from the same promoter. The failure of the Pro115 mutant proteins to recognize curved DNA and to bend DNA despite their near normal affinity for non-curved DNA can be attributed to a defect in protein-protein interaction resulting in a reduced capacity to form oligomers observed *in vitro* and by a new *in vivo* test based on functional replacement by H-NS of the oligomerization domain (C-domain) of bacteriophage λ cI repressor.

Keywords: curved DNA/DNA bending/DNA binding proteins/protein-protein interaction in vivo/transcriptional repression

Introduction

H-NS is one of the most abundant proteins associated with the nucleoid of Enterobacteriaceae. Several lines of evidence indicate that this protein, in addition to participating in the structural organization of the bacterial chromosome, is also involved in the regulation, primarily at the transcriptional level, of a select and fairly large number of genes (for reviews see Higgins *et al.*, 1990; Ussery *et al.*, 1994). The structural basis for H-NS function seems to reside in the capacity of this protein to recognize and bind specifically to intrinsically curved regions of DNA with little sequence specificity (Bracco *et al.*, 1989; Yamada *et al.*, 1990, 1991; Owen-Hughes *et al.*, 1992; Falconi *et al.*, 1993; Zuber *et al.*, 1994).

Some years ago, we demonstrated that hyperproduction of *Escherichia coli* H-NS strongly inhibited both transcription and translation and caused the nucleoids to become extremely compact and almost perfectly spherical, ultimately resulting in cell death (Spurio *et al.*, 1992). To serve as a control for these studies, with the hope of obtaining a protein molecule inactive in DNA binding, we constructed an *hns* mutant lacking four in-frame codons (hence the name *hns*Δ12) corresponding to the tetrapeptide G112-R113-T114-P115, which is close to the conserved Trp108 residue involved in protein-DNA interaction (Friedrich *et al.*, 1988; Tippner and Wagner, 1995). Indeed, overproduction of this protein proved to be non-toxic and did not perturb the above-mentioned macromolecular synthesis. Nevertheless, hyperproduction of the mutant protein resulted in a less pronounced, yet clearly detectable, condensation of the nucleoid and the resulting protein could be retained by matrix-bound DNA up to a fairly high concentration of NaCl. These findings suggested that, although somewhat different from that of the wild-type, the DNA binding capacity of the mutant protein was not, or at least not entirely, lost (Spurio *et al.*, 1992) and prompted us to pursue further the study of H-NSΔ12 and of similar mutants with the hope of shedding light on the mechanism by which H-NS specifically recognizes curved DNA.

In this article we present data indicating that, in addition to recognizing curved DNA, wild-type H-NS can also induce curvature in non-bent DNA and that both functions are lost in H-NSΔ12 and in mutants in which Pro115 is either deleted or replaced by alanine. These mutants were shown to have preserved an intact basal DNA binding activity but to have an impaired capacity to oligomerize *in vitro* and *in vivo*. Our results lead to the conclusion that the recognition of curved DNA and the bending of non-curved DNA depend on the quaternary structure of H-NS.

Results

Construction of H-NS mutants in the 112-115 region

According to the three-dimensional structure of the C-terminal portion of the H-NS molecule recently

elucidated by NMR spectroscopy (Shindo *et al.*, 1995), the G-R-T-P tetrapeptide deleted in H-NSΔ12 corresponds to the distal portion of loop 2, which connects the anti-parallel β-sheet containing the Trp108 implicated in DNA binding (Friedrich *et al.*, 1988; Tippner and Wagner, 1995) and the C-terminal α-helix (Figure 1A). In the light of the interesting biological properties of the H-NSΔ12 mutant described above, we first investigated if the deletion or substitution of only one of the four amino acids missing in H-NSΔ12 could account for the phenotype of this mutant. Thus, the following mutations, obtained by single base substitutions, were introduced in H-NS: Arg113→His, Thr114→Ser and Pro115 →Ala; in addition, a deletion of Pro115 (ΔPro115) was produced. Mutations of Gly112 gave rise to proteins giving non-reproducible phenotypes and therefore these mutants will not be considered here.

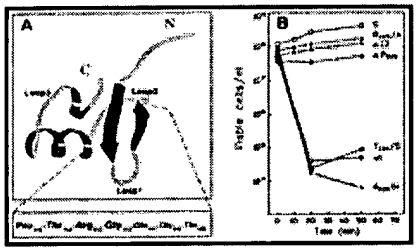

Fig. 1. Localization of the peptide containing Pro115 within the three-dimensional structure of H-NS and toxic effect of hyperproduction of H-NS molecules bearing mutations in this region. (**A**) Three-dimensional structure of the C-terminal domain (47 residues) of H-NS as elucidated by Shindo *et al.* (1995) by two-dimensional NMR spectroscopy showing loop 2 which contains the Gly112-Arg113-Thr114-Pro115 tetrapeptide deleted in the previously described H-NSΔ12 mutant (Spurio *et al.*, 1992) and representing the target of the mutagenesis carried out in the present work. (**B**) Effect of overproduction of wild-type and mutant H-NS molecules on cell viability. The experiment was carried out essentially as described (Spurio *et al.*, 1992). The DNA fragments containing the indicated *hns* mutants were cloned into expression vector pPLc2833 (Remaut *et al.*, 1983) and transformed into *E.coli* HB101 carrying plasmid pcI encoding the temperature-sensitive cI857 λ repressor. Overproduction of wild-type and mutant H-NS was carried out as previously described (Spurio *et al.*, 1992). Open square, control (uninduced) cells; cells expressing: open circle, wild-type H-NS; open triangle, H-NSΔ12; closed circle, H-NSΔPro115; open triangle, H-NSPro115Ala; closed square, H-NSThr114Ser; closed diamond, H-NSArg113His.
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In vivo toxicity of the H-NS mutants

In agreement with our previous report (Spurio *et al.*, 1992), hyperproduction of wild-type H-NS caused a decrease in the viable cell counts by at least three orders of magnitude within 20 min of induction; cells hyperproducing comparable amounts of H-NSΔ12 did not display any loss of viability, thus behaving, at least from this point of view, like the control cells carrying the expression vector without insert (Figure 1B). As seen from the same figure, hyperproduction of the two Pro115 mutant proteins (i.e. Pro115 →Ala and ΔPro115) had no toxic effect on the cells, while the Arg113 and Thr114 mutant proteins were as toxic as the wild-type (Figure 1B). Considering the similarity in behaviour between the two Pro115 mutant proteins and H-NSΔ12, we pursued further the functional characterization of these two Pro mutant proteins. Since the phenotypes displayed by the two mutant proteins were found to be essentially identical in all tests performed, in some cases only results obtained with ΔPro115 will be shown.

Protein-protein interaction in vivo

Preliminary characterization of H-NSΔ12 indicated that this protein displayed a DNA binding behaviour different from that of the wild-type. Thus, when subjected to chromatography on DNA-cellulose columns, both proteins displayed rather heterogeneous elution patterns consisting of two main peaks: one, predominant in cells overproducing the mutant protein with a background of wild-type H-NS, eluted at lower ionic strength (250 mM NaCl); the other, prevailing in cells producing only the wild-type protein, eluted at higher ionic strength (350 mM NaCl) (Figure 2A). Upon rechromatography, the wild-type H-NS eluted as a homogeneous peak at 350 mM NaCl, regardless of whether it was eluted in the first or second peak from the first column; in contrast, the material containing H-NSΔ12 eluted from the second DNA-cellulose column at an ionic strength corresponding to that at which it was originally eluted (Figure 2B). We interpreted these results to mean that the different elution patterns reflect different aggregation states of the protein and that the wild-type and mutant H-NS molecules differ in their capacity to oligomerize. Indeed, when the wild-type and mutant H-NS were compared by gel filtration, the results suggested that the mutant molecules might be defective in protein-protein interaction resulting in an impaired capacity to form tetramers.

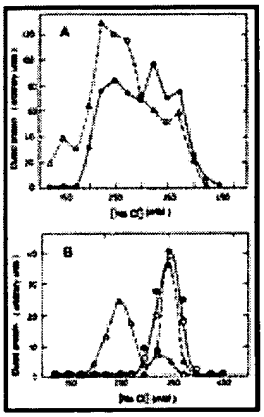

Fig. 2. Chromatographic behaviour of wild-type H-NS and H-NSΔ12 on matrix-bound DNA. *Escherichia coli* K12Δ*H1*Δ*trp* cells harbouring either pPLc11 or pPLc12 (Spurio *et al.*, 1992) were grown in M9 minimal medium supplemented with glucose, thiamine, ampicillin and all non-radioactive amino acids with the exception of methionine, which was added as the ^{35}S-labelled amino acid (Falconi *et al.*, 1996). After induction to hyperproduce wild-type H-NS or H-NSΔ12 (Spurio *et al.*, 1992), the proteins were purified essentially as described (Falconi *et al.*, 1988). (**A**) Primary chromatography. Wild-type [^{35}S]H-NS (closed circle) or [^{35}S]H-NSΔ12 (open triangle) were loaded onto DNA-cellulose and eluted with a linear NaCl gradient as described (Falconi *et al.*, 1988). The eluted material was subjected to SDS-PAGE and the resulting gel slabs were dried, autoradiographed and the amount of H-NS quantified by densitometry of the autoradiograms. (**B**) Secondary chromatography. The material eluting between 200 and 300 mM (open circle, open triangle) and between 300 and 400 mM (closed circle, closed triangle) NaCl from the primary chromatograph of wild-type H-NS (open circle, closed circle) or H-NSΔ12 (open triangle, closed triangle) was rechromatographed under conditions identical to those used for the primary chromatography.
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To investigate this point in more detail with the Pro115 mutant proteins, a test to determine the actual capacity of the H-NS molecules to oligomerize *in vivo* was devised. Thus, starting with plasmid pBF21, which carries the bacteriophage λ *cI* repressor under the control of a tandemly repeated *lac*UV5 promoter, we deleted the DNA encoding the oligomerization domain of the repressor (C-terminal domain), producing pBF22, and, from this, we prepared a series of constructs (pBF23-pBF26) encoding chimeric proteins consisting of the N-terminal domain of the λ cI repressor and of wild-type or mutant H-NS molecules. The construct pBF27, expressing a chimeric protein consisting of the N-terminal domain of the λ repressor fused to *E.coli* translation initiation factor IF1 (Figure 3A), and pBF28, expressing a defective cI repressor fused to wild-type H-NS, served as controls. The rationale of the

experimental approach is schematically illustrated in Figure 3B; the test relies on the capacity of an oligomerization-proficient protein domain to functionally replace the natural C-terminal domain of phage λ cI repressor conferring biological activity (i.e. transcriptional repression of phage λ early promoters) to the N-terminal DNA binding domain of the same repressor. The oligomerization proficiency of a protein can be quantitated by determining the number of lytic plaques formed following infection (with phage λ) of *E.coli* cells harbouring the pBF expression vectors and producing, upon induction with IPTG, the various types of chimeric λ cI repressor molecules (Figure 3A). The time course of phage λ production, expressed as p.f.u./ml, following λ infection of the IPTG-induced cells carrying the various constructs is presented in Figure 3C and D. In cells expressing the entire wild-type cI repressor, the number of plaques formed is drastically lower (~8 orders of magnitude) compared with those expressing only the N-terminal domain of the repressor or the chimeric molecule containing a typically monomeric protein such as translation initiation factor IF1 (Figure 3C). Wild-type H-NS, on the other hand, can replace, to a large extent, the function of the C-terminal domain of the cI repressor and substantially reduce the lytic growth of phage λ (Figure 3D); this effect can only be due to an H-NS-induced oligomerization of the DNA binding domain of the repressor, since a non-specific interference of H-NS with the lytic cycle of phage λ is ruled out by the finding that wild-type H-NS fused to a defective N-terminal domain of the cI repressor, as in the construct pBF28, is completely unable to repress phage growth (Figure 3C). Furthermore, when fused through its N-terminus, H-NS apparently loses its DNA binding capacity, since it can be overexpressed by the cells without any toxic effect (not shown). Finally, any non-specific effect of H-NS on the lytic cycle of λ phage seems to be ruled out by the finding that both kinetics of propagation and burst size are virtually identical in two *E.coli hns* alleles, namely HMG9 (Goransson *et al.*, 1990) and YK4124 (Yazusawa *et al.*, 1992), and in their respective isogenic strains (not shown).

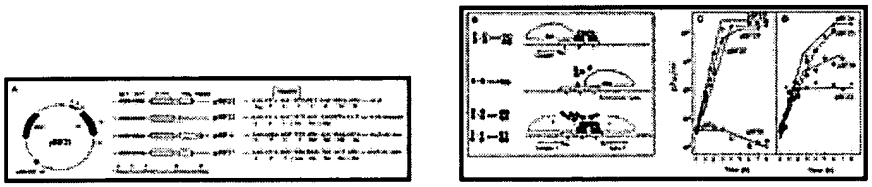

Fig. 3. *In vivo* protein oligomerization assay. (**A**) Schematic representation of plasmid pBF21 encoding, under the control of tandem *lac*UV5 promoters (*lacP*), the native λ *cI* repressor containing both the DNA binding (N-terminal) domain (vertically striped area) and the oligomerization (C-terminal) domain (white box). In the other constructs, this dimerization domain is deleted (pBF22) or replaced by *infA** (pBF27), by wild-type *hns* or by mutant *hns* (pBF*). pBF* represents any one of the following constructs: pBF23, wild-type H-NS; pBF24, H-NSΔ12; pBF25, H-NSPro115Ala; pBF26, H-NS ΔPro115. The relative positions of *Eco*RI (E) and *Hin*dIII (H) restriction sites and sequences of the constructs at the *cI* fusion sites are also indicated. The amino acid residues are indicated by the one letter code in the case of cI protein and by the three letter code in the case of H-NS and IF1. Residues introduced during the cloning procedure are shown in parentheses. (**B**) Schematic illustration of the principle of the assay as described in the text. Cells harbouring the different constructs shown in (A) were induced to express wild-type or chimeric λ cI repressor molecules by addition of IPTG. Following infection of these cells with phage λ, lytic development of the phage is prevented in cells producing wild-type cI repressor, which binds cooperatively to the DNA operator sites by virtue of protein-protein

interactions involving the C-terminal domain of the protein. Cells producing a truncated repressor molecule lacking the C-terminal domain undergo a complete lytic cycle, producing a rapid burst of phages. The fate of the cells producing chimeric λ cI repressor molecules depends on the capacity of the fused protein to induce oligomerization of the truncated cI repressor. (**C** and **D**) Number of plaques (p.f.u./ml) of λ phages released at the indicated times after infection of *E.coli* cells harbouring: open square, pBF21; open circle, pBF22; closed triangle, pBF23; open diamond, pBF24; closed square, pBF25; closed circle, pBF26; open triangle, pBF27; closed diamond, pBF28. Further details are given in Materials and methods.
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When this test was applied to the H-NS mutants, it was found that H-NSΔ12 and H-NSPro115Ala had completely lost the capacity to prevent lytic growth of λ, while H-NSΔPro115 was severely impaired in this activity (Figure 3D). Aside from the Pro115 mutants, none of the >10 different H-NS mutants produced by site-directed mutagenesis in our laboratory has shown any defect in functional replacement of the oligomerization domain of the λ cI repressor. This is a further indication of the specificity of this test and of the defect caused in H-NS caused by Pro115 mutations. Thus, we conclude that the protein-protein interaction necessary for oligomerization of H-NS is impaired whenever Pro115 is either deleted or replaced.

Protein-protein interaction in vitro

As mentioned above, H-NSΔ12 had shown a different elution profile in gel filtration experiments, suggesting an oligomerization defect in this mutant protein. A comparison of the gel filtration patterns of wild-type H-NS and H-NSΔPro115 is shown in Figure 4. As seen from this figure, compared with the wild-type, the amount of protein eluting in the 59 kDa and the 28 kDa peaks (corresponding to the molecular weights of tetramers and dimers) is reduced and the amount of material eluting as a monomer of 13.6 kDa is increased in the profile of the mutant H-NS. Furthermore, compared with wild-type H-NS, the first two peaks of the mutant are somewhat broadened and their positions are shifted to larger elution volumes, indicating that the aggregated forms of the H-NSΔPro115 molecule are more prone to dissociate during the course of gel filtration. The results of these experiments are in full agreement with the conclusions, drawn from the preliminary experiments carried out with H-NSΔ12 and with the *in vivo* results presented above, that the mutant H-NS is defective in oligomerization.

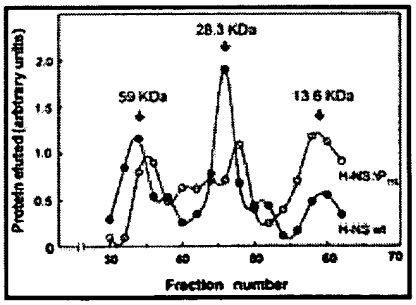

Fig. 4. Analysis of the oligomerization state of wild-type H-NS and mutant H-NSΔPro115 by gel filtration. A Sephadex G75 column (60×2 cm) equilibrated in 20 mM Tris-HCl, pH 8.0, buffer containing 0.5 mM EDTA, 2.5% glycerol, 200 mM NaCl, 5 mM β-mercapto-ethanol was used. Before loading, wild-type H-NS (closed circle) and mutant H-NSΔPro115 (open circle) were preincubated for 20 min at 20°C at 1 μM concentration in 2 ml elution buffer; the proteins were then eluted at 20°C at a flow rate of 1 ml/min and 2.1 ml fractions were collected. To allow detection of the minute amounts of protein eluting from the column, 300 μl of each fraction were transferred onto sheets of nitrocellulose using the Bio-Dot apparatus (BioRad) and exposed to rabbit polyclonal anti-H-

NS antiserum. The filters were subsequently developed with peroxidase-conjugated anti-rabbit serum and the intensity of bands was quantified by imaging densitometer (BioRad model GS-670). The indicated molecular weights were deduced from the elution volumes of the following molecular weight standards used to calibrate the column: *E.coli* translation initiation factor IF1 (8.1 kDa); myoglobin (17.2 kDa); soya bean trypsin inhibitor (20.1 kDa); ovalbumin (43 kDa); bovine serum albumin (66.2 kDa).
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Pro115 H-NS mutants fail to interact with a naturally curved DNA site and to repress transcription

The mechanism of transcriptional autorepression by H-NS is fairly well understood at the molecular level and can be regarded as a model of at least one type of mechanism by which H-NS may interfere with transcription. In this case, inhibition is mediated by the binding of H-NS to two extended regions flanking a static bend mapped by circular permutation at ~-150 and to a lower affinity site partly overlapping the -35 element of the *hns* promoter (Falconi *et al.*, 1993). The results presented below show that both physical and functional interaction of H-NS with this natural target are lost in Pro115 mutants.

The gel shift experiments presented in Figure 5 clearly show that, compared with wild-type H-NS, the affinity of H-NSΔPro115 for the intrinsically bent DNA fragment is severely reduced (Figure 5A and B); more importantly, the electrophoretic behaviour of the retarded DNA fragment depends on the type of protein with which it interacts (Figure 5A), suggesting that the wild-type and mutant H-NS form different types of complexes with the same DNA fragment. This premise is further supported by the results of the DNase I footprinting experiment, which indicate that specific protection of the curved DNA target is observed with wild-type H-NS but not with the Pro115 mutant (Figure 5C).

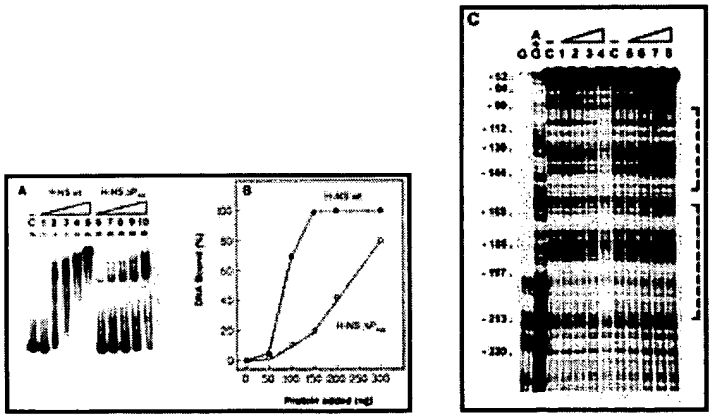

Fig. 5. Binding of wild-type H-NS and H-NSΔPro115 to a naturally curved DNA fragment. (**A**) Electrophoretic band shift of the intrinsically curved 207 bp *Eco*RI fragment of the *hns* promoter representing the upstream regulatory element of *hns* transcriptional autorepression (Falconi *et al.*, 1993). Lane C, no protein added; lanes 1-5 and 6-10, 50, 100, 150, 200 and 300 ng wild-type H-NS and H-NSΔPro115, respectively. The figure shows the electrophoretically separated ^{32}P-labelled bands as detected by a BioRad Molecular Imager (model GS-250). (**B**) Percentage of total DNA bound by H-NS,

quantified by Molecular Imager, plotted as a function of the amount of protein added. (**C**) Limited DNase I digestion pattern of the 207 bp fragment derived from the *hns* promoter region in the presence of increasing amounts of wild-type H-NS (lanes 1-4) or H-NSΔPro115 (lanes 5-8). Lane C, digestion in the absence of protein; the amounts of protein added in 20 μl reaction mixtures were (lanes 1-8): 0.3, 0.6, 0.8, 1, 0.6, 1.2, 1.6 and 2.2 μg respectively. Lanes G and G+A represent Maxam-Gilbert sequencing lanes. DNA regions protected from DNase I by wild-type H-NS are indicated by vertical broken lines. Experimental conditions for both gel shift and DNase I footprinting were essentially as previously described (Falconi *et al.*, 1993, 1996).
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Transcription *in vitro* of a reporter gene fused to the *hns* promoter fragment containing the naturally curved DNA segment recognized by H-NS was also studied. Thus, we looked at inhibition of transcription in the presence of increasing amounts of either wild-type H-NS or of H-NSΔPro115 after a fixed time of incubation (Figure 6A) and at the time course of transcription in the presence of a fixed amount of either protein (Figure 6B). The results clearly demonstrate that inhibition of transcription caused by the ΔPro115 mutant is substantially reduced compared with that caused by wild-type H-NS. Furthermore, as mentioned above, inhibition by wild-type H-NS is accompanied by a specific protection of the promoter region from digestion with DNase I, while the mutant H-NS does not give any specific footprints at concentrations comparable with those at which it inhibits transcription (Figure 5C). This clearly indicates that, while transcriptional inhibition by wild-type H-NS is mediated by an interaction of the protein with the specific curved DNA target, the reduced transcriptional inhibition caused by the mutant H-NS molecule must be due to a non-specific interaction with DNA.

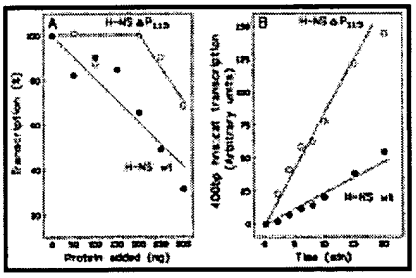

Fig. 6. Effect of wild-type H-NS and H-NSΔPro115 on transcriptional activity of the *hns* promoter. *In vitro* transcription was performed essentially as previously described using as template plasmid pKK400, which contains a promoterless *cat* gene under the control of the promoter and upstream regulatory region of *hns* (Falconi *et al.*, 1993). Transcription products were analysed by Northern blotting and probed using a radiolabelled *cat* gene. Signals were detected and quantified by BioRad Molecular Imager. Transcriptional activity, expressed as a function of (**A**) increasing amounts of protein and (**B**) the time of incubation at 32°C in the presence of 250 ng protein. Closed circle, wild-type H-NS; open circle, H-NSΔPro115.
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The preferential interaction of wild-type H-NS with curved DNA is strongly diminished in Pro115 mutants

In addition to the intrinsically curved DNA sites representing its natural targets, H-NS can also bind to synthetic curved and non-curved DNA fragments having the same base composition, showing a high specificity for curved fragments (Yamada *et al.*, 1990, 1991). To determine whether or not the H-NSPro115 mutants were impaired in their capacity to select a synthetic curved DNA fragment, we

performed competitive band shift experiments. For this purpose, we synthesized the same model curved (A_5N_5) and non-curved (A_5N_{10}) DNA fragments (Koo *et al.*, 1986) previously used by Yamada *et al.* (1990). As seen in Figure 7A, a non-curved DNA fragment is not as efficient as a curved fragment in chasing a radioactive curved DNA fragment bound to wild-type H-NS. This confirms the known preference of this protein for binding to curved DNA. In comparison, H-NSΔPro115 had lost a large portion of this binding selectivity in favour of the curved fragment; this was partly due to the fact that the non-curved fragment had become a better competitor and partly because the curved DNA fragment had become a less efficient competitor (Figure 7B). As a result, if the capacity of H-NS to discriminate between curved and non-curved DNA is calculated by comparing the amount of each competitor DNA (bent or non bent) required to chase 50% of a preformed H-NS-DNA complex, this discrimination drops from >10-fold in favour of curved DNA found with wild-type H-NS to <3-fold found in the case of H-NSΔPro115.

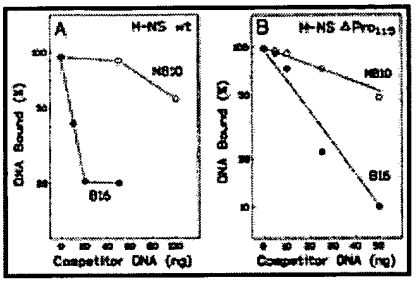

Fig. 7. Diminished binding selectivity of mutant H-NSΔPro115 towards curved DNA. Curved (B16) and non-curved (NB10) DNA fragments were prepared as described in Materials and methods and an aliquot of B16 was labelled with [^{32}P]dATP by fill-in reaction. For the competitive band shift retardation experiments, 5 ng portions of the radioactive DNA fragment were incubated in 15 µl 10 mM Tris-HCl, pH 8.0, containing 10 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, 1 mM spermidine, 0.5 mM dithiothreitol and 5% glycerol with the indicated amounts of either non-radioactive NB10 (open circle) or non-radioactive B16 (closed circle) DNA fragments and with either 100 ng wild-type H-NS (**A**) or 200 ng H-NSΔPro115 (**B**). After 30 min at 30°C the samples were subjected to band shift assay and the amount of radioactive curved DNA remaining bound to protein was quantified with a BioRad Molecular Imager.
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Wild-type H-NS can bend non-curved DNA, while Pro115 mutants cannot

While it is well established that H-NS can preferentially bind to curved DNA, no evidence exists so far that this protein can also induce bending of non-curved DNA. In the next series of experiments, we investigated this particular point by ligase-mediated circularization experiments. The rigidity of the DNA duplex does not allow spontaneous circularization of DNA fragments whose length is shorter than the persistence length (P = 150 bp) and limits the rate of ring formation for DNA fragments up to ~500 bp. For our experiments, we have chosen a strictly non-curved DNA fragment of 155 bp based upon the A_5N_{10} sequence described by Koo *et al.* (1986). As seen from the electrophoretic separation presented in Figure 8A, an increasing amount of this linear DNA fragment becomes circular upon incubation with increasing concentrations of wild-type H-NS in the presence of phage T4 DNA ligase. From the quantification of these results, it is clear that the level of circularization of the fragment in the presence of wild-type H-NS reaches a substantially higher level than that obtained in the presence of the ligase alone (Figure 8B). Under the specific conditions of this experiment, ~5% of the total DNA was circularized in the absence of H-NS, while the circular form reached a plateau at ~20% of the total in the

presence of ~100 ng protein. In contrast to wild-type H-NS, H-NSΔPro115 was totally inactive in this DNA circularization test and increasing amounts of this mutant protein actually caused a small reduction in the amount of circular form produced by the ligase alone. Similar conclusions can be reached from the results obtained in the experiment in which the time course of circularization was measured in the presence and absence of wild-type and mutant H-NS. As seen in Figure 8C-F, DNA circularization increased with time, reaching ~25% of the total after 5 h in the presence of wild-type H-NS, while H-NS ΔPro115 proved again to be inactive in this test.

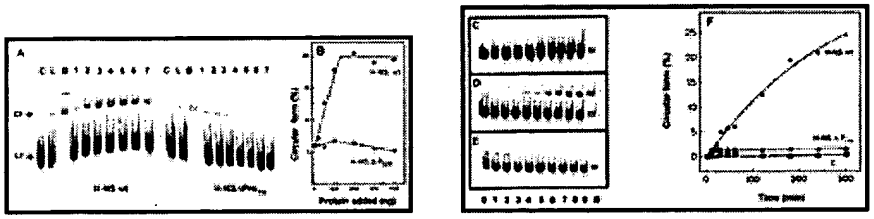

Fig. 8. DNA ligase- and H-NS-dependent circularization of non-curved DNA. Preparation of the substrates for T4 ligase-dependent DNA cyclization and conditions for the circularization assay are as described in Materials and methods. (**A**) Electrophoretic separation of the ligation products of DNA NB10 in the presence of increasing amounts of protein. Lanes 1-7, 20, 50, 100, 200, 300, 400 and 800 ng wild-type H-NS or H-NSΔPro115. Lane C, DNA incubated in the absence of proteins; lane L, DNA incubated with T4 DNA ligase in the absence of H-NS; lane B, DNA resistant to *Bal*31 nuclease digestion. LF and CF indicate the positions of the linear and circular forms of DNA, respectively. (**B**) Percentage circular DNA produced in the presence of wild-type H-NS (closed circle) and H-NSΔPro115 (open circle) was quantified by Molecular Imager and plotted as a function of the amount of protein added. Time course of DNA circularization in the absence of H-NS (**C**) or in the presence of 400 ng either wild-type H-NS (**D**) or H-NSΔPro115 (**E**). The DNA circularization reaction was carried out as described in Materials and methods, incubating for the following times: 0 (0), 10 (1), 20 (2), 30 (3), 45 (4), 60 (5), 120 (6), 180 (7), 240 (8) and 300 min (9). Lane B, DNA resistant to *Bal*31 digestion. Percentage circular DNA produced in the absence of H-NS (closed square) or in the presence of either wild-type H-NS (closed triangle) or H-NSΔPro115 (open triangle) was quantified by Molecular Imager and plotted as a function of time of incubation (**F**).
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When similar tests were carried out using a curved DNA fragment of 165 bp based upon the A_5N_5 sequence described by Koo *et al.* (1986), wild-type H-NS produced only a marginal stimulation of the circularization reaction, while the Pro115 mutants inhibited the reaction only very slightly (not shown). This result suggests that wild-type H-NS does not contribute further bending to this DNA fragment, which is already substantially curved. Furthermore, the finding that the mutant H-NS causes only a very slight reduction in the level of circularization of both bent and non-bent DNA fragments obtained in the presence of the ligase alone seems to rule out the possibility that the failure of the mutant protein to promote DNA circularization might be due to induction of non-coherent bends. In fact, if this hypothesis were correct, one would expect strong inhibition of the `basal' circularization reaction in the presence of the mutant protein, which was not found.

Finally, the failure of wild-type H-NS to stimulate circularization of bent DNA and the finding that wild-type H-NS did not stimulate religation of restriction fragments obtained from pBR322 (not shown) indicate that circularization of the non-curved 155 bp DNA fragment by wild-type H-NS was not due to an effect of this protein on activity of the DNA ligase but must be due to bending of the DNA substrate. Thus, we conclude from our experiments that while wild-type H-NS can actively induce bending in non-curved DNA, the Pro115 mutants are inactive in this function.

Discussion

The present study stems from the observation that deletion of a tetrapeptide comprising Pro115 produced a mutant H-NS protein (H-NSΔ12) with unexpected properties; this mutant protein retained substantial DNA binding activity, but its hyperproduction, unlike that of the wild-type protein, was found not to inhibit macromolecular syntheses, was not toxic to cells and caused a less drastic compaction of the nucleoid (Spurio *et al.*, 1992). We have now subjected *E.coli hns* to site-directed mutagenesis in the region coding for this peptide and have shown that deletion or replacement with alanine of Pro115 alone is sufficient to produce molecules with properties similar or identical to those of the original H-NSΔ12 mutant. These Pro115 mutant proteins have also been found to retain almost normal affinity for non-curved DNA, but to have, compared with the wild-type, a substantially reduced preference for synthetic curved DNA. Furthermore, the mutant proteins failed to recognize the intrinsically curved DNA fragment located at -150 in the *hns* promoter (Falconi *et al.*, 1993), as indicated by their failure to produce the DNase I protection pattern characteristic of the wild-type and by their reduced efficiency in transcriptional autorepression.

Nucleoid protein H-NS binds to DNA with little sequence specificity (Rimsky and Spassky, 1990; Lucht *et al.*, 1994), preferentially recognizing intrinsically curved DNA structures (Bracco *et al.*, 1989; Yamada *et al.*, 1990; Owen-Hughes *et al.*, 1992; Falconi *et al.*, 1993; Zuber *et al.*, 1994). H-NS induces compaction of the DNA (Spassky *et al.*, 1984; Spurio *et al.*, 1992) and generates negative supercoiling (Owen-Hughes *et al.*, 1992; Tupper *et al.*, 1994). Through these functions H-NS plays the dual role of contributing to the structural organization of the bacterial chromatin and of controlling expression of a fairly large number of genes (Bertin *et al.*, 1990; Higgins *et al.*, 1990; Yoshida *et al.*, 1993; Ussery *et al.*, 1994). The molecular mechanism by which these activities are accomplished and the nature of the structural alteration imposed on the DNA by this protein have so far remained largely obscure; for instance, although one could expect that a protein with preference for curved DNA would also be able to alter the helical axis of the duplex (Kahn and Crothers, 1992), active bending of DNA by H-NS had never been demonstrated. Furthermore, our knowledge concerning the structure of H-NS is very limited, the available information concerning its three-dimensional structure being restricted to a low resolution model of its C-terminal domain (Shindo *et al.*, 1995) and none of the several spontaneous *hns* alleles isolated so far has helped to shed light on the nature of the active sites of the protein.

In the present article, we demonstrate that, in addition to binding preferentially to naturally curved DNA, wild-type H-NS can also promote ligase-mediated circularization of a 155 bp non-curved DNA

fragment. Since it is known that the relative stiffness of short segments of the DNA double helix (<500 bp) necessitates bending or kinking to bring otherwise distant segments into close proximity (Wang and Giaever, 1988; Travers, 1995), our results demonstrate for the first time that H-NS, like HU, IHF, Fis and Lrp (Thompson and Landy, 1988; Hodges-Garcia *et al.*, 1989; Wang and Calvo, 1993; Nash, 1996) can also actively bend DNA.

Furthermore, our results have shown that the Pro115 mutants, in parallel with their failure to recognize intrinsically curved DNA, have also lost the capacity to bend non-curved DNA. Thus, through the use of the Pro115 mutants, we have demonstrated that the DNA bending activity and the capacity to recognize naturally curved DNA are activities which can be separated from the intrinsic DNA binding capacity of the protein and strongly depend on its oligomerization. This situation is reminiscent of other DNA binding proteins (e.g. the *lac* repressor) whose regulatory function is largely dependent on their oligomerization property (Chakerian and Matthews, 1992).

To reveal the oligomerization defect of the Pro115 mutants, we have developed a test which can monitor and quantitatively assay the strength of protein-protein interaction *in vivo*. In this system, the C-terminal domain, responsible for oligomerization of phage λ cI repressor, was substituted by either H-NS wild-type, H-NS Pro115 mutants or, as a control, with translation initiation factor IF1, a typically monomeric nucleic acid binding protein. The capacity of these chimeric constructs to oligomerize, thus preventing lytic growth of the phage, is reflected in the number of phages produced. When this test was applied, the constructs containing wild-type H-NS or several H-NS mutants bearing different amino acid replacements (not shown) were able to functionally replace the wild-type λ cI repressor; only the Pro115 and the H-NSΔ12 mutants were unable to oligomerize.

As shown in Figure 1A, Pro115 is located in the C-terminal domain of H-NS, the portion of the molecule for which the three-dimensional structure has been determined. This domain is considered to encompass at least part of the DNA binding activity of H-NS in that it binds to DNA, albeit with an affinity which is three to four orders of magnitude lower than that of the intact molecule (Shindo *et al.*, 1995). The C-terminal domain of H-NS contains an α-helix and an anti-parallel β-sheet separated by a loop where Pro115 is located. Deletion or substitution of Pro115 may alter the mutual orientation of these secondary structure elements, resulting in defective protein-protein interaction. Only complete elucidation of the three-dimensional structure of H-NS will allow clarification of this point, however.

Recent data from our laboratory (unpublished results) have shown that H-NS mutants of the conserved Trp108 residue, implicated in DNA binding by fluorescent spectroscropic studies (Friedrich *et al.*, 1988; Tippner and Wagner, 1995), have DNA binding properties similar to those of the Pro115 mutants (i.e. intact basal DNA binding property and severely reduced capacity to recognize curved DNA and to bend DNA), but display a normal oligomerization behaviour.

The essential role of both Trp108 and H-NS oligomerization for recognition of curved DNA and for bending DNA suggests a simple model by which H-NS may perform these activities. According to this model, two or more H-NS dimers may bind to DNA by virtue of their intrinsic DNA binding affinity, possibly displaying a preference for AT-rich regions (Rimsky and Spassky, 1990; Lucht *et al.*, 1992).

As in the case of other DNA binding proteins (Werner *et al.*, 1996), the conserved tryptophan residue of H-NS may perturb the course of the helical axis of the duplex by intercalation or through other types of hydrophobic interactions, creating foci of DNA bending. The resulting curvature would then become stabilized by long range protein-protein interaction between H-NS dimers.

This model is consistent with the bimodal distribution of H-NS footprints on both sides of naturally curved DNA targets (Falconi *et al.*, 1993), with the cooperative nature of its interaction with these sites (Rimsky and Spassky, 1990; Falconi *et al.*, 1993; Ueguchi and Mizuno, 1993; Zuber *et al.*, 1994) and, more generally, with the fairly large body of evidence suggesting that both transcriptional repression and compaction of the nucleoid by H-NS require two not mutually exclusive mechanisms. These are linear polymerization of the protein on DNA and DNA looping through interaction between H-NS molecules bound to separated primary binding sites (Zuber *et al.*, 1994, and references therein).

Materials and methods

Construction of H-NS mutants

The *hns* gene, cloned into pSelect, was subjected to site-directed mutagenesis following the `Altered site *in vitro* mutagenesis' protocol (Promega). Arg113, Thr114 and Pro115 were modified using the following oligonucleotides: 5′-dCCAAGGCCATACTCCAG-3′, 5′-dGGCCGTAGTCCAGCTGT-3′ and 5′-dCCGTACTGCAGCTGTAA-3′. Pro115 was deleted using the same procedure with an oligonucleotide having the sequence 5′-dAGGCCGTACTGCTGTAATCA-3′. The mutations were confirmed by DNA sequencing and, in the case of the deletion mutant, by restriction analysis, since this mutation causes the loss of the unique *Pvu*II site in *hns*.

Preparation of curved and a non-curved DNA fragments

Preparative ligation of the synthetic oligonucleotides 5′-dGGCAAAAACG-3′ and 5′-dCCCGTTTTTG-3′ and 5′-dCCGGCAAAAACGGGC-3′ and 5′-dGGGCCCGTTTTTGCC-3′, respectively corresponding to the A_5N_5 and A_5N_{10} sequences described by Koo *et al.* (1986), was carried out as described by Yamada *et al.* (1990). The resulting multimers were blunt-ended by fill-in reaction with Klenow fragment and cloned into the unique *Sma*I site of pTZ18 (Pharmacia). Two of the resulting plasmids (i.e. pB16 and pNB10, where B = bent and NB = non-bent and the number indicates the number of tandem repeats of the 10mer or 15mer) were selected. From the two plasmids two *Eco*RI-*Hin*dIII restriction fragments (i.e. B16 of 230 bp and NB10 of 220 bp) were obtained.

Preparation of DNA cyclization substrate

A 235 bp DNA fragment containing NB10 was amplified by PCR using primers with the following sequences: 5′-dGTAAAACGACGGCCAGT-3′ and 5′-dGGAATTCGAGCTCGGATCCC-3′. The second primer allowed replacement of the *Kpn*I site of the pTZ18 polylinker with a *Bam*HI site which, upon cleavage, yielded a non-curved 155 bp DNA fragment. Following purification by agarose gel

electrophoresis, electroelution and dephosphorylation with calf intestinal phosphatase (CIP), the fragment was end-labelled with [γ-^{32}P]ATP in the presence of phage T4 polynucleotide kinase. A similar procedure was used to amplify the B16 sequence and prepare a 165 bp curved DNA fragment.

DNA cyclization reaction

Aliquots of ~5×10^{-2} pmol labelled DNA were incubated in 20 µl 50 mM HEPES buffer, pH 8, containing 50 mM potassium glutamate, pH 8, 10 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, 33 µg/ml bovine serum albumin and the indicated amounts of the specified proteins. After 15 min at 20° C, phage T4 DNA ligase was added (0.26 U/20 µl reaction tube) and, unless otherwise specified, incubation was continued for 2 h. Where necessary, samples were treated with *Bal*31 nuclease (0.033 U) in the presence of 12 mM $CaCl_2$. At the end of the incubation, the reactions were stopped by addition of 10 µl of a solution containing 100 mM EDTA, 6 mg/ml proteinase K, 0.25% SDS, 15% glycerol and heated at 55°C for 20 min. The ligation products were analysed by electrophoresis at 12 V/cm on 6% polyacrylamide non-denaturing gels. Dried gels were quantified using a BioRad Molecular Imager (model GS-250).

Construction of phage λ cI repressor fusions

A 1.7 kbp phage λ DNA fragment containing the *cI* gene under the control of a tandem *lac*UV5 promoter-operator region was excised from pKB252 (Backman *et al.*, 1976) by complete digestion with *Eco*RV and partial digestion with *Eco*RI and ligated into pBR322 cut with *Eco*RI and *Eco*RV to generate pBF21. Plasmid pBF22, which encodes only the first 159 amino acids of the cI repressor, was constructed from pBF21 by removal of the 866 bp *Hin*dIII-*Eco*RV fragment, filling in the protruding ends and inserting, by blunt end ligation, SMURFT oligonucleotides (Pharmacia) coding for amber stop codons in all three translational frames. pBF23 was made by a two step cloning procedure. First, the proximal region of *hns* was modified making use of complementary oligonucleotides 5′-dATTTTAAGTGCTTCGCTCATAAAGCTTG-3′ and 5′-dAATTCAAGCTTTATGAGCGAAGCACTTA-3′ to create a *Hin*dIII site just upstream of the ATG translational start codon. Second, a *Hin*dIII-*Dra*I fragment containing the entire gene and the transcription termination sites was moved from pTZ18, used as an intermediate vector for DNA manipulations, and cloned into the *Hin*dIII and *Eco*RV sites of pBF21 to generate a chimeric gene encoding for a fusion polypeptide consisting of 160 amino acids of the cI repressor and 136 amino acids of H-NS. The constructs pBF24, pBF25 and pBF26 are identical to pBF23, but for the fact that the *hns* fused to the *cI* repressor carries the Δ12, Pro115→Ala and ΔPro115 mutations, respectively. pBF28 is a derivative of pBF23 where the *cI* repressor gene was partially deleted by removal of the DNA region between *Nsi*I and *Hin*dIII sites. The correct frame was restored using two synthetic oligonucleotides (5′-dAGCTTGGCTTGGATCCTGTTGGTGATGCA-3′ and 5′-dTCACCAACAGGATCCAAGCCA-3′) to produce a shortened cI, missing 96 amino acid residues (Leu57-Ala152) fused to the wild-type *hns* gene. To obtain pBF27, which contains a fusion between the proximal portion of *cI* and *infA**, pXR101 (Calogero *et al.*, 1987) was digested with *Eco*RI and *Acc*I to excise a fragment which was substituted by two synthetic oligonucleotides (5′-dAATTCGGAAGCTTAGGGT-3′ and 5′-dATACCCTAAGCTTCC-3′) to create a *Hin*dIII site. Excision of the 240 bp *Hin*dIII-*Sph*I fragment and

its subsequent cloning into pBF21 digested with the same restriction enzymes then allowed the in-frame insertion of the first AUG codon of *E.coli* translation initiation factor IF1. Details of the DNA region surrounding the *Hin*dIII site within the *cI* gene and *cI* fusion constructs are shown in Figure 3A.

Protein-protein interaction in vivo

Escherichia coli 71-18 *lacI*^{q} cells (Yanish-Perron *et al.*, 1985), transformed with the constructs described above, were grown at 37°C to an $A_{620\ nm}$ of 0.6 in LB medium containing ampicillin. The production of chimeric proteins consisting of the N-terminal (DNA binding) domain of λ cI repressor fused to the desired protein was induced by addition of 250 µl 100 mM IPTG to 0.4 ml portions of the culture. Following this induction, the cells were infected by addition of 3 µl phage λ 146 (5×10^{10} p.f.u./ml) (Bailone and Devoret, 1978) diluted 1/10 000 just before infection in 10 mM Tris-HCl, pH 8.0, buffer containing 10 mM $MgSO_4$. After 5 min incubation at 20°C, 5 ml LB containing ampicillin (100 µg/ml) and 10 mM $MgSO_4$ were added to the infected cultures which were transferred to a 37°C shaker bath. At the indicated times, 0.4 ml aliquots were removed, centrifuged for 5 min to pellet the cells and 150 µl supernatant were mixed with 30 µl chloroform. The number of phages in each supernatant was determined by the quantitative titer method of Silhavy *et al.* (1984) using *E.coli* 71-18 as recipient strain.

Footnotes

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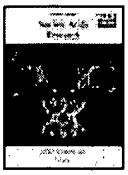

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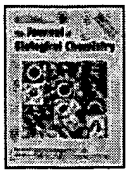

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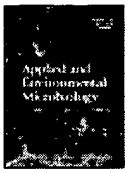

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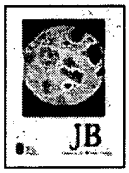

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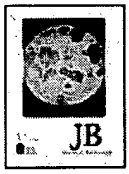

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